Antibodies and labeling reagents

Directly labeled anti-CD3 (clone SK7), -CD107a (H4A3) -CD11c (B-ly6), -CD56 (B-159), -CD86 (IT2.2), -CD94 (HP-3D9), -CD14 (M5E2), -CD19 (SJ25C1) and –IFN-γ (B27) were from BD Biosciences. Anti-HLA-ABC (W6/32) was from AbdSerotec. Anti-CD16 (3G8) was from Invitrogen. Anti-CD83 (HB15a), -CD158b1/b2,j (clone GL183, KIR2DL2, KIR2DL3, and KIR2DS2), -CD158a,h (clone EB6B, KIR2DL1, and KIR2DS1), were from Beckman Coulter. Anti–HLA-DR (L243) and -IFN-γ (4S.B3) were from Biolegend. Anti-CD1c (AD5-8E7) was from Miltenyi Biotech. Unconjugated goat polyclonal anti–IL-15Rα and anti-IL-12 were from R&D Systems. Anti-α-tubulin (AA13) was from Sigma. Blocking antibodies XA141 (anti-CD158a/p58.1 and -CD158h/p50.1, IgM), Y249 (anti-CD158b1/p58.2 and -CD158j/p50.2, IgM), DEC66 (anti-CD158k, IgM) and A6136 (anti-HLA class I, IgM) were a gift from G. Ferlazzo, University of Messina, Italy. Anti–HLA-ABC (W6/32) and anti-HLA-DR (L243) monoclonal antibodies were from Biolegend. Anti-WASP (clone5A5) was from BD Biosciences. Anti-IL12R2 and anti-KIR3DL1 polyclonal antibodies were from SantaCruz and anti-Perforin (MAB4616) was from Chemicon. Cells labeled with unconjugated antibodies were stained with Alexa Fluor 555–conjugated rabbit anti-mouse IgG, Alexa Fluor 647–conjugated rabbit anti-mouse IgG, Alexa Fluor 555–conjugated donkey anti-goat, Alexa Fluor 647-conjugated goat anti-mouse IgM, Alexa Fluor 568-conjugated goat anti-mouse IgG2a(γ2a) (all from Invitrogen), or Rhodamine Red-X–conjugated donkey anti-goat (Jackson ImmunoResearch Laboratories). F-actin staining was performed with bodipy conjugated phallacidin (Invitrogen). For Western Blot experiments HRP-conjugated goat anti-mouse (Biorad) and mouse anti-Vinculin (Sigma) were used. Control antibodies for stainings were normal goat IgG (R&D Systems), mouse IgG1 (MOPC-21) from BioLegend, mouse IgG2a,κ (eBM2a) from eBioscience and mouse IgM from Invitrogen. Live and

dead cell staining in flow cytometry experiments was performed with aqua fluorescent reactive dye from Invitrogen.

Human DCs and NK cells

PBMCs were isolated from leukocyte concentrates (New York Blood Center and Zürich Blood Center) by density-gradient centrifugation on Ficoll/Hypaque. $CD14^+$ cells were isolated from PBMCs by positive magnetic cell separation (Miltenyi Biotec) and cultured for 5 days in R2 (RPMI1640 supplemented with 2% heat-inactivated pooled human AB serum (Invitrogen)), IL-4 (Peprotech), and GM-CSF (Invitrogen), according to standard protocols [1]. $CD14^-$ fraction or freshly isolated NK cells were frozen. Monocyte-derived DCs were matured for 36-48 hours in R2 supplemented with IL-4, GM-CSF, with 25µg/ml of poly(I:C) or poly(I:C)-LMW (Invivogen) or with a cocktail of pro-inflammatory cytokines (10ng/ml IL-1β, 10ng/ml TNFα, 1000U IL-6 (R&D Systems) and 1μg/ml PGE2 (Sigma)). Maturation of monocyte-derived DCs was monitored by flow cytometry using anti-CD11c, -CD83, -CD86, -HLA-DR and –HLA-ABC antibodies. Blood DCs were isolated from PBMCs by positive magnetic cell separation using the CD1c $(BDCA\text{-}1)^+$ dendritic cell isolation kit (Miltenyi Biotec). Blood DCs were cultured overnight in R2, GM-CSF and 25 µg/ml of poly(I:C). Purity and maturation of blood DCs was monitored by flow cytometry using CD1c, CD14, CD19, CD83, CD86, HLA-DR and HLA-ABC specific antibodies. NK cells were isolated from frozen or fresh $CD14^-$ or $CD19^-CD1c^-$ fractions by negative selection using the NK cell isolation kit II (Miltenyi Biotec). Resulting purity of $CD3^-CD56^+CD16^{+/-}$ cells was higher than 97% of total cells and less than 2% were T cells, as determined by flow cytometry. Peripheral blood NK cells subsets were separated from total blood NK cells by positive selection with CD16 microbeads (Miltenyi Biotec) When specified, NK cells were activated for 5 days with 500U of IL-2 (Peprotech)

K562 cells and their HLA class I transfection

K562 cells were maintained in RPMI 1640 medium (GIBCO BRL, Grand Island, NY) supplemented with penicillin (10 IU/ml), streptomycin (100 µg/ml) and 10% FCS (GIBCO BRL). Cell cultures were kept in a water-saturated atmosphere with 5% CO_2 at 37°C.

For the generation of HLA-Bw4-expressing K562 cells, K562 cells were transfected with HLA-B*3701 cDNA, respectively, inserted into the expression vector pcDNA3.1 (Invitrogen, Groningen, Netherlands). Briefly, K562 cells were transfected by electroporation with plasmid DNA (10 µg/1x10^6 cells) in OPTIMEM1 with Glutamax (GIBCO BRL). Electroporation conditions were 250V, 300 µF using the Gene Pulser ® II (BioRad, München, Germany). Transfected cells were selected by the addition of G418 (1 mg/ml) to the culture medium and subsequently cloned by single cell sorting of HLA-positive cells using flow cytometry.

Stealth^{TM} siRNA duplexes and electroporation of DCs

Stealth^{TM} siRNA duplexes were purchased from Invitrogen. SiRNA sequences targeting WASP were 5'AGGAGCUGUACUCACAGCUUGUCUA3' and 5'UAGACAAGCUGUGAGUACAGCUCCU3' (sense and antisense respectively). Electroporation of immature monocyte-derived DCs with siRNA targeting WASP or control siRNA was performed as described before [2]. Briefly, 4 x 10^6 immature DCs were resuspended in Opti-MEM medium without phenol red (Invitrogen) and electroporated with 1nmol of *si*RNA duplexes, in 4mm cuvettes, in a ECM830 Square Porator TM with a unique square wave pulse 500V 0.5ms. Electroporated DCs were plated and matured with poly(I:C), as described before.

Human mature DC/resting NK cell co-cultures and conjugation assays

Resting or IL-2 activated NK cells were cultured with autologous mature monocyte-derived DCs or autologous mature blood DCs, at a ratio of 2:1 for 6h or 24h at 37°C in R5 or R10 (RPMI1640 supplemented with 5% heat-inactivated pooled human AB serum or 10% FCS) and gentamicin. For quantification of conjugates by flow cytometry, NK cells were pre-stained with the PKH26

dye (Sigma). Stained NK cells were washed 3 times in R5, transferred to a clean tube and allowed to rest at 37ºC for at least 1 hour to inhibit passive transfer of the PKH26 dye to the DC in DC/NK cell co-cultures. DCs were stained with a Pacific Blue-coupled, anti-HLA-DR antibody (due to it's uniform and bright staining on mature DCs), washed 3 times and co-cultured with PKH26-stained NK cells for different time points. When specified, DMSO or cytochalasin B (10μM, Sigma) were added. After conjugation, cells were resuspended with 3% PFA and fixed for 20min at 4ºC. Where specified, fixed BFA-treated co-cultures were permealized and intracellular IFN-γ was stained. Before FACS acquisition, cells were washed and resuspended in PBS. In blocking experiments, mature DCs or resting NK cells were cultured for 1h on ice with isotype matching antibodies or blocking antibodies before co-culture. F-actin polymerization inhibitor or DMSO were added at either 0 or 2h after mixing of the cells. For flow cytometry experiments, labeled anti-CD107a antibody was added at the beginning of the co-culture and brefeldin A (BFA 5μg/ml, Sigma) was added after 1 hour of co-culture. 5 hours later, cells were fixed, permeabilized and stained for flow cytometry with antibodies and fixable Aqua. For accessing susceptibility of DCs to NK-cell-mediated killing, DC single cultures or DC/NK cell co-cultures were stained with ToPro3 (Invitrogen). Where indicated, DCs (bottom) were separated from NK cells (top) by 0.4μm pore membranes (Corning). For synapse assays, resting NK cells were cultured with mature autologous or allogeneic monocyte-derived DCs or mature blood DCs. Cells were mixed at a NK cell/DC ratio of 2:1 in 20μl of RPMI1640 without serum, prior to being pelleted quickly at 10'000g. When specified, DMSO or cytochalasin B were added. The pellets were then allowed to conjugate for 0 to 30min at 37°C. Cells were resuspended in RPMI1640 and centrifuged onto poly-lysine–coated 1.5-mm coverslips for immunofluorescence analysis. Where indicated, resting NK cells were co-cultured with K562 cells or with HLA-Bw4 transfected K562 cells, in a ratio of 2:1, following similar conditions as the DC/NK cell co-cultures.

DC protein extracts and Western Blot

Mature DCs were collected and washed once in PBS. Cells were then resuspended in ice cold lysis buffer (NP-40 1% with complete protease inhibitor cocktail from Roche), vortexed for 10sec and incubated on ice for 10min. Cell lysates were centrifuged for 10min at 4ºC at 13'000g, and protein concentrations were quantified with the BCA protein assay (Pierce). For all samples, 50μg of total protein extracts were boiled for 5 min in the presence of SDS-PAGE-loading buffer (250mM Tris-HCl pH 6.8, 40% glycerol, 8% SDS, 0.57M β-mercaptoethanol, 0.12% bromophenol blue). Boiled extracts were loaded on 10% SDS-PAGE gels and transferred onto a PVDF membrane (Hybond-P, Amersham Biosciences). For detection of primary antibodies, HRP-conjugated goat anti-mouse IgG (Biorad) and the ECL plus detection system (Amersham Biosciences) were used. For quantification of protein levels, the Immobilon Western Chemiluminescent HRP Substract kit from Millipore was used for detection of primary antibodies and images were acquired in a Fujifilme Intelligent Dark Box with a LAS-3000 camera. Quantification of band intensity was performed by Image J, on pictures without pixel-saturation.

Live cell imaging

Mature DCs and autologous resting NK cells were washed separately and stained with CSFE (Molecular Probes) and PKH26, respectively. Stained DCs and NK cells were washed 3 times in R5 without phenol red, transferred to a clean tube and allowed to rest at 37ºC for at least 1 hour to inhibit passive transfer of the dyes in the cocultures. Cells were resuspended in R5. Labeled DCs were added to a basement membrane matrix, growth factor reduced, phenol red-free matrigel (BD Biosciences) coated chamber, as recommended by the manufacturer. Labeled NK cells were added to the DCs, in a proportion of 2 NK cells to 1 DC, and image acquisition was performed immediately after. Cocultures were visualized through a x63 1.4 NA oil immersion lens with an inverted CLSM Leica SP5 Mid UV-VIS confocal microscope for at least 90min. Co-cultures were kept at 37ºC and 5% CO_2 during the co-culture experiment and image acquisition.

Transmission electron microscopy

Co-cultures of mature DCs, mature *ΔWASP* DCs, mature *control* DCs and K562 cells with resting NK cells were pelleted at 16'000g for 1min and fixed in 0.8% formaldehyde, 2.5% glutaraldehyde solution in 0.05 M cacobuffer for 1h at room temperature. Pellets were washed with 0.05 M cacobuffer and second fixation was done with a solution of 2% OsO4 and 3% potassium ferrocyanide 1:1 for 1h, and washed again. Pellets were embedded in 2.5% agar, dehydrated with a gradient of alcohol from 70% to 100% and further Epon embedded. Polymerization was performed at 60ºC for 3 days. Semi-thin sections of 1µm were obtained, stained with toluidine blue and ultra-thin cuts of 70nm were obtained. Ultra-thin cuts were collected on grids and stained with uranylacetate and Reynold's Lead Citrate Solution. Cells were visualized with a Philips CM100 microscope and images acquired with a Gaton Orius CCD Camera.

Immunofluorescence microscopy and analysis

Cells on slides were fixed in 3% PFA for 20min at 4°C. Cells were permeabilized with 0.01% Triton-X for 1 min at room temperature. Cells were then incubate with the Image-iT FX signal enhancer (Invitrogen) and stained with the indicated antibodies followed by the appropriate secondary reagent. All washes were performed in PBS supplemented with 1% fish skin gelatin (Sigma-Aldrich) and 0.02% saponin (Sigma-Aldrich). After staining, slides were counterstained with DAPI and mounted with Prolong gold anti-fade reagent (Invitrogen). Slides were visualized through a ×100 1.4 NA oil immersion lens with an inverted Olympus IX-70 microscope (DeltaVision Image Restoration Microscope; Applied Precision/Olympus) and a Photometrics CoolSnap QE camera, or with an inverted Leica LX microscope and a Leica DFC 350 FX camera. Serial optical sections (0.2 µm; 40-60 sections) were acquired for all labelings. Images were deconvoluted using DeltaVision SoftWoRx software version 3.4.4. or Huygens software 1.1.4 (Montpellier RIO Imaging). Fold enrichment values were determined by evaluation of at

least 100 conjugates in randomly selected fields in at least 3 separate experiments. Mature DC/resting NK cell conjugates were identified using: (a) chromatin density, in which DAPI staining stains brightly condensed and round-shaped nuclei of NK cells as opposed to less dense DC nuclei; (b) cytoplasm area, in which DCs have a large area of cytoplasm and the ratio between cytoplasm and nucleus is small in NK cells; and (c) f-actin staining, by which cell-cell interaction can be properly visualized, cytoplasm can be identified and time of interactions can be selected on the basis of f-actin polymerization at the synapse. Quantification of molecules at the synapse in conjugates was done after image analysis with ImageJ software version 1.41n (NIH). We measured the enrichment of a molecule at the contact site of each conjugate compared with the distribution of the same molecule in a similar area opposite to the contact site within the same cell. The fold enrichment was calculated as the average intensity per unit volume at the contact site or at the opposite membrane across the contact site divided by the average intensity per unit volume of the entire cell. The values obtained in unconjugated cells were considered as baseline for statistics, and data represent the ratio of relative enrichment at the contact site to relative enrichment at the membrane across the contact site normalized to the values in single cells, assigned as 1. For analysis of NK-cell MTOC or perforin granules, conjugates that presented these structures in the radial area formed from the center of the conjugated NK cell to the bounderies of the immunological synapse were considered as polarized. For synapse analysis, stacks were rebuilt, and orthogonal views of the z-stack were visualized with Image J. Analyses evaluating the distribution and colocalization of voxels within the reconstructed synapse were made using JACoB in ImageJ. Analisis of live cell imaging were performed with Imaris 7 software (Bitplane).

REFERENCES

1. Münz C, Dao T, Ferlazzo G, de Cos MA, Goodman K, Young JW. Mature myeloid dendritic cell subsets have distinct roles for activation and viability of circulating human natural killer cells. *Blood*. 2005;105(1):266-273.

2. Breton G, Yassine-Diab B, Cohn L, et al. siRNA knockdown of PD-L1 and PD-L2 in monocyte-derived dendritic cells only modestly improves proliferative responses to Gag by $CD8^+$ T cells from HIV-1-infected individuals. *J Clin Immunol*. 2009;29(5):637-645.

Figure S1. $CD56^{bright}CD16^{-}$ NK cells conjugate more readily with mature DCs and *K562/resting NK cell conjugates display rapid NK cell-derived f-actin polymerization at the synapse*

(A) Mature *DCs* and resting $CD56^{bright}CD16^{-}$ or $CD56^{dim}CD16^{+}$ NK cells were pre-stained with vital dyes and co-cultured for 1, 20 or 120min. The percentage of NK cell-conjugated DCs was determined by flow cytometry.

(B) Cocultures between resting $CD56^{bright}CD16^{-}$ or $CD56^{dim}CD16^{+}$ NK cells and autologous mature DCs were fixed after 1 or 20min of interaction. F-actin (green), nuclear DNA (blue) and α-tubulin (red) were stained. Arrows point to the NK cell-MTOC. The left graph represents the quantification of f-actin staining intensity at the synapse, compared with the staining at the opposite side of the same DC. Values were normalized to the values of molecule distribution in unconjugated cells, assigned as 1. The number of conjugates with MTOCs of NK cells distant to the synapse were plotted in the right graph, as percentages of total number of conjugates analyzed. Statistics were performed with a null hypothesis of 75%, representing random distribution in the non-polarized quadrants.

(C) Resting NK cell single cell cultures or K562/resting NK cell co-cultures were fixed after 1 or 20min of interaction and f-actin was stained with bodipy-conjugated phallacidin (green). DAPI was used to stain the nuclear DNA (blue). Arrows indicate the synapse. The graph represents the quantification of f-actin staining intensity at the synapse, compared with the staining at the opposite side of the same NK cell. Values were normalized to the values of molecule distribution in unconjugated cells, assigned as 1.

(D) K562 cells were allowed to conjugate with resting NK cells for 20min. Co-cultures were fixed and stained with bodipy-conjugated phallacidin to stain f-actin (green) and anti-KIRs antibodies (red). The fluorescence intensities of KIRs and f-actin stainings were plotted along the indicated trajectory, from A to B. The synapse area in cellular conjugates is indicated by boxes.

(E) K562 cells were allowed to conjugate with resting NK cells for 20min. Co-cultures were fixed and treated for TEM analysis. Original magnifications are 3'400x, 13'500x and 66'000x, respectively, from left to right. . The graph represents the size of several regions in the synaptic clefts of 5 K562/NK cell conjugates. The images are representative of 1 experiment for TEM and at least 3 independent experiments for the other techniques. Original magnifications are 100x for all the light microscopy images. Values on graph bars are medians and error bars represent interquartile ranges from the analysis of at least 100 conjugates from at least 3 independent experiments. ↑↑ Fold increase > 2; *p<0.05. P values from Mann-Whitney test, non-parametric and bi-caudal. Scale bars are 10μm unless specified otherwise.

Figure S2. NK cell-*MTOC polarization and f-actin polymerization at the cytotoxic synapse between K562 cells and resting NK cells is controlled by MHC class I/KIR inhibitory signaling.*

(A) K562/resting NK cell co-cultures were fixed after 1 or 20min of interaction. F-actin (green), nuclear DNA (blue) and α-tubulin (red) were stained. Arrows point to the NK cell MTOC. The number of conjugates with MTOCs of NK cells adjacent to the synapse was plotted in the graph as percentages of total number of conjugates analyzed.

(B) K562 and HLA-Bw4 expressing K562 cells were stained with HLA-ABC or isotype control antibodies. Cells were gated as live, individual cells. ΔMFI: delta mean fluorescence intensity.

(C) K562-Bw4 cells cocultures with resting KIR3DL1^{+} or KIR3DL1^{-} NK cells were fixed after 20min of incubation. F-actin (green), nuclear DNA (blue), α-tubulin (red) and KIR3DL1 (white) were stained. Arrows point to the NK cell MTOC. The top graph represents the quantification of f-actin staining intensity at the synapse, compared with the staining at the opposite side of the same NK cell. Values were normalized to the values of molecule distribution in unconjugated cells, assigned as 1. The number of conjugates with MTOCs of NK cells adjacent to the synapse were plotted in the bottom graph, as percentages of total number of conjugates analyzed.

Original magnifications are 100x for all the microscopy images. A total of at least 200 conjugates from 3 independent experiments were analyzed. Values on graph bars are medians and error bars represent interquartile ranges. ↑↑ Fold increase >2; *p<0.05 Statistics were performed with a null hypothesis of 25%, representing random distribution in the polarized quadrant. Scale bars are 10μm.

Figure S3. ***IL-12 and MHC class II polarizations at the synapse are not detectable in poly(I:C) matured DC/resting NK cell conjugates.***

(A) Poly(I:C) or pro-inflammatory-cytokine matured DC single cell cultures or mature DC/resting NK cell co-cultures were fixed after 20min of interaction and F-actin (green), nuclear DNA (blue) and IL-12 (red) were stained. Arrow points to IL-12 enrichment at the synapse.

(B) Poly(I:C) matured DC single cell cultures or mature DC/resting NK cell co-cultures were fixed after 1 or 20min of interaction and F-actin (green), nuclear DNA (blue), MHC class I (white) and MHC class II (red) were stained. Arrows point to molecules enrichment at the synapse. Graphs represent the quantification of IL-12 **(A)**, MHC class I or MHC class II **(B)** staining intensity at the synapse, compared with the staining at the opposite side of the same DC. Values were normalized to the values of molecule distribution in unconjugated cells, assigned as 1. Images are representative of at least 3 **(A)** or 2 **(B)** independent experiments. Original magnifications are 100x for all the microscopy images. Values on graph bars represent medians from the analysis of at least 100 conjugates **(A)** or 60 conjugates **(B)** from at least 3 **(A)** or 2 **(B)** independent experiments. Error bars indicate interquartile ranges. ↑ Fold enrichment >1.5;↑↑ fold enrichment >2. Scale bars are 10μm.

Figure S4. HLA mismatched DC/NK cell conjugates present cytotoxic features

(A) Mature *control DCs* or mature *ΔWASP* DCs and resting NK cells were pre-stained with vital dyes and co-cultured for 6h, in the presence of BFA, and IFN-γ production by unconjugated NK cells (marked by the red box) was assessed by ICS in flow cytometry.

(B) Co-cultures of HLA-Bw4 mismatched mature DCs with resting NK cells were fixed after 20min of interaction. F-actin (green), nuclear DNA (blue), tubulin or MHC class I (red) and KIR3DL1 (white) were stained. Arrows point to molecule enrichment at the synapse. * points to the NK cell MTOC. The number of conjugates with NK cell-derived MTOCs polarization at the synapse was plotted in the left graph, as percentages of total number of conjugates analyzed. The right graph represents the quantification of f-actin and MHC class I staining intensity at the synapse, compared with the staining at the opposite side of the same conjugated cell. Values were normalized to the values of molecule distribution in unconjugated cells, assigned as 1. Images are representative at least 3 independent experiments. Original magnifications are 100x for all the light microscopy images. Values on graph bars are medians and error bars represent interquartile ranges from the analysis of at least 100 conjugates from at least 3 independent experiments. ↑↑ Fold enrichment >2; *$p<0.05$. Statistics were performed with a null hypothesis of 25%, representing random distribution in the non-polarized quadrant. Scale bars are 10μm.

Figure S5. ***F-actin polymerization is essential for maturation of the mature DC/resting NK cell synapse***

(A) Cytochalasin B or DMSO were added to co-cultures of mature DCs with resting NK cells, after 0 or 20min of interaction and conjugates were fixed after 20 or 30min of interaction. F-actin (green) and nuclear DNA (blue) were stained. The graph represents the quantification of f-actin staining intensity, at the synapse, compared with the staining at the opposite side of the same conjugated DC. Values were normalized to the values of f-actin distribution in unconjugated DCs, assigned as 1.

(B) Mature DCs and resting NK cells were pre-stained with vital dyes and co-cultured for 120min. DMSO (control) or cytochalasin B were added to the co-cultures after 0 or 20 min of interaction. The percentage of NK cell-conjugated DCs was determined by flow cytometry following the gating strategy indicated in the left.

(C) Mature DCs and resting NK cells were pre-stained with vital dyes and co-cultured for 6h, in the presence of BFA, and IFN-γ production by unconjugated NK cells (marked by the red box) was assessed by ICS in flow cytometry. DMSO (control) or cytochalasin B were added to the co-cultures after 0 or 2h of interaction (C0 and C2 respectively).

(D) NK cells were cultured alone, for 6h, in the presence of BFA and of PMA/Ionomycin or just medium, and IFN-γ production by NK cells was assessed by ICS, followed by flow cytometry. DMSO and cytochalasin B were added at 0h after starting of culture. NK cells were gated as live, individual, $CD3^{-}CD56^{+}$cells.

(E) Mature DCs and resting NK cells were co-cultured, for 6 hours, in the presence of DMSO or cytochalasin B, and the percentage of live and dead cells was assessed by flow cytometry, using Fixable Aqua Live/Dead reagent. Cytochalasin B was added at 0h or 2h after starting of culture (C0 and C2, respectively). Images are representative of at least 3 independent experiments. Original magnifications are 100x for all the microscopy images. Values on graph bars **(A)** represent medians from the analysis of at least 100 conjugates. Values on graph bars **(B, C and E)** represent medians from 3 independent experiments with duplicates. Error bars indicate interquartile ranges. ↑↑ Fold increase >2, ** $p<0.001$, *** $p<0.0001$. P values from Mann-Whitney test, non-parametric and bi-caudal. Scale bars are 10μm.

Figure S6. F-actin dynamics control IFN-γ production by NK cells in a cell-contact dependent manner and stabilize spatial distribution of MHC class I molecules at the synapse.

(A) NK cells were cultured alone or with mature DCs, for 24h, and IFN-γ production by NK cells was assessed by ELISA. DMSO and cytochalasin B were added at 0h or 2h after starting of culture (C0 and C2, respectively). n.d.: not detectable.

(B) As in **(A)** but cells were separated by a solvent-permeable transwell, where specified. n.d.: not detectable.

(C) DMSO or cytochalasin B were added to co-cultures of mature DCs with resting NK cells and conjugates were fixed after 20min of interaction. F-actin (green), nuclear DNA (blue), IL-15Rα (white) and MHC class I (red) were stained. Z-stacks at synapse (red dotted line) were rebuilt and colocalization of molecules was measured. Arrows point to molecule enrichment at the synapse and arrowhead points to colocalized molecules at the synapse. Images are representative of at least 3 independent experiments. Original magnifications are 100x for all the microscopy images. Values on graph bars **(A and B)** represent medians from 3 independent experiments with duplicates. Values on graph bars **(C)** represent medians from the analysis of at least 15 conjugates from 3 independent experiments. Error bars indicate interquartile ranges. **p<0.001 and ***p<0.0001. P values from Mann-Whitney test, non-parametric and bi-caudal. Scale bars are 10μm.

Figure S7. ***Inhibition of f-actin polymerization does not increase IFN-γ production during NK cell recognition of a MHC class I deficient target***

(A) NK cells were cultured with K562 for 6 hours, in the presence of BFA and IFN-γ production by NK cells was assessed by ICS, followed by flow cytometry. DMSO or cytochalasin B were added at 0h or 2h after starting of culture (C0 and C2, respectively). NK cells were gated as live, individual, $CD3^{-}CD56^{+}$cells.

(B) As in **(A)** but culture time was 24 hours without the presence of BFA. IFN-γ was measured by ELISA. Plots are representative of at least 3 independent experiments. Values on graph bars represent medians from at least 3 independent experiments with duplicates. Error bars indicate

interquartile ranges.

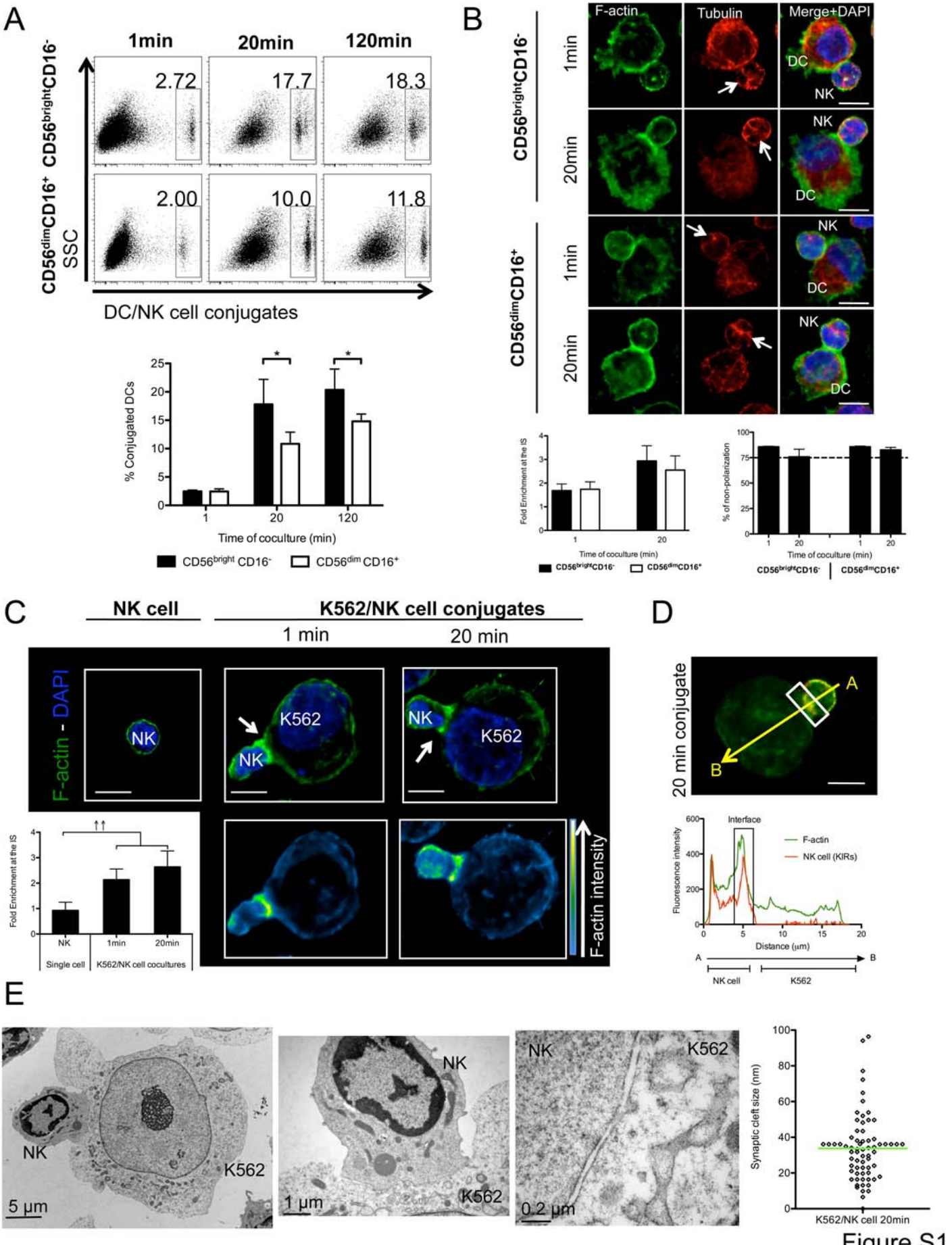
A
1min
20min
120min
2.72
17.7
18.3
2.00
10.0
11.8
CD56brightCD16-
CD56dimCD16+
SSC
DC/NK cell conjugates
% Conjugated DCs
Time of coculture (min)
CD56bright CD16-
CD56dim CD16+
B
F-actin
Tubulin
Merge+DAPI
DC
NK
Fold Enrichment at the IS
% of non-polarization
C
NK cell
K562/NK cell conjugates
1 min
20 min
F-actin - DAPI
K562
F-actin intensity
Single cell
K562/NK cell cocultures
D
20 min conjugate
Interface
F-actin
NK cell (KIRs)
Fluorescence intensity
Distance (μm)
NK cell
K562
E
5 μm
1 μm
0.2 μm
Synaptic cleft size (nm)
K562/NK cell 20min

Figure S1

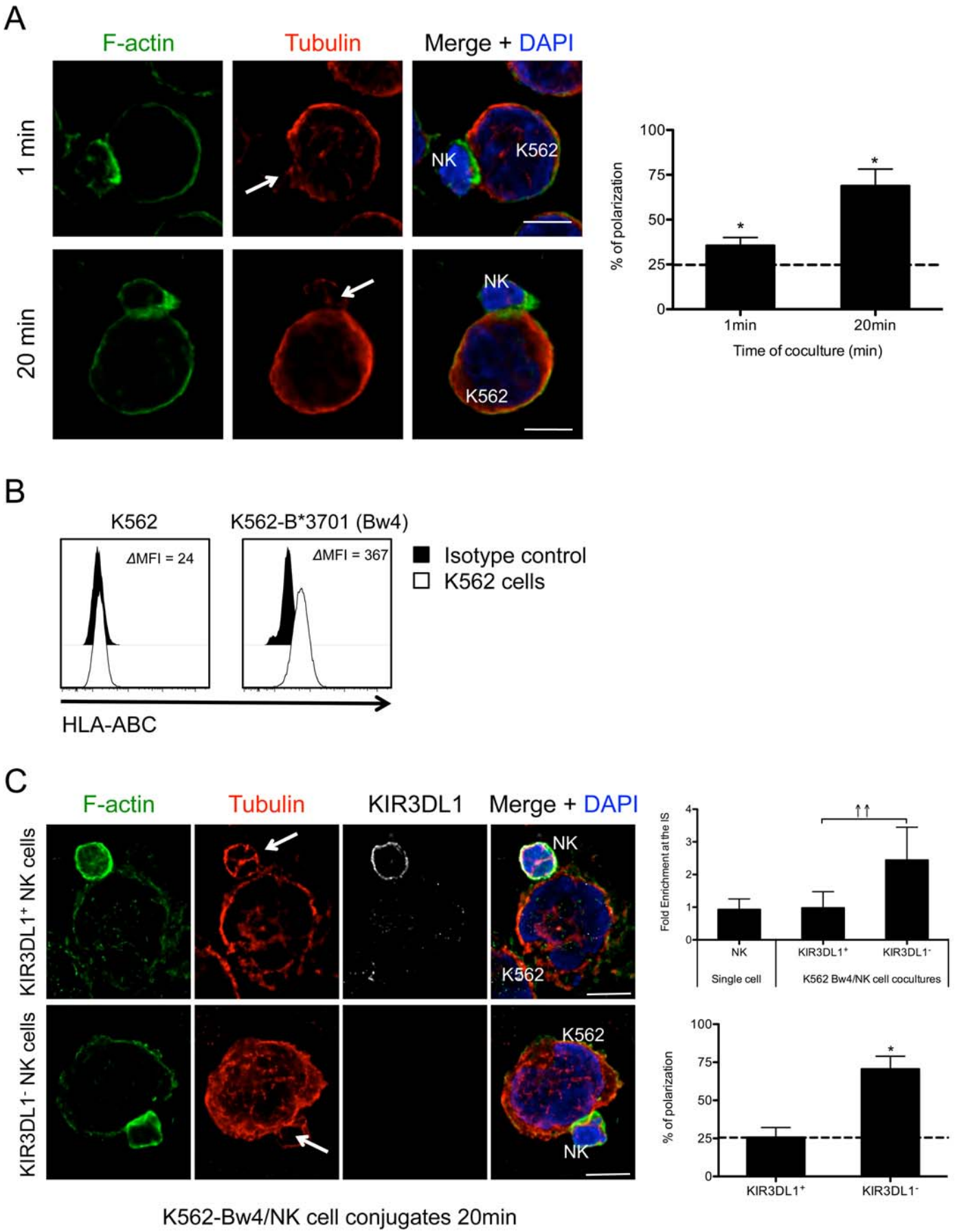
A
F-actin
Tubulin
Merge + DAPI
1 min
20 min
NK
K562
% of polarization
100
75
50
25
0
1min
20min
Time of coculture (min)
B
K562
K562-B*3701 (Bw4)
ΔMFI = 24
ΔMFI = 367
Isotype control
K562 cells
HLA-ABC
C
F-actin
Tubulin
KIR3DL1
Merge + DAPI
KIR3DL1+ NK cells
KIR3DL1- NK cells
Fold Enrichment at the IS
NK
KIR3DL1+
KIR3DL1-
Single cell
K562 Bw4/NK cell cocultures
K562-Bw4/NK cell conjugates 20min

Figure S2

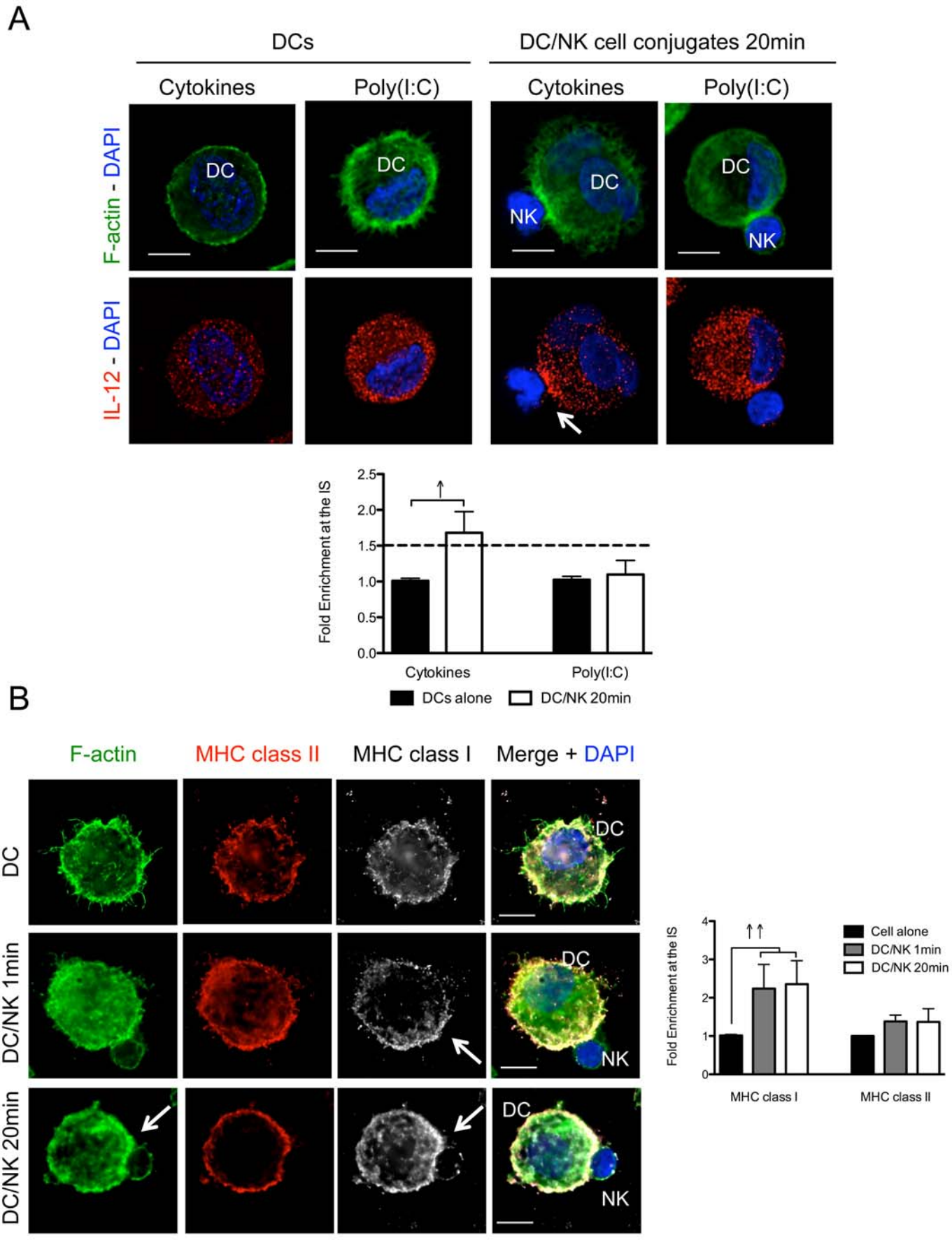
A
DCs
DC/NK cell conjugates 20min
Cytokines
Poly(I:C)
Cytokines
Poly(I:C)
F-actin - DAPI
IL-12 - DAPI
DC
NK
Fold Enrichment at the IS
2.5
2.0
1.5
1.0
0.5
0.0
Cytokines
Poly(I:C)
DCs alone
DC/NK 20min
B
F-actin
MHC class II
MHC class I
Merge + DAPI
DC
DC/NK 1min
DC/NK 20min
Fold Enrichment at the IS
4
3
2
1
0
Cell alone
DC/NK 1min
DC/NK 20min
MHC class I
MHC class II

Figure S3

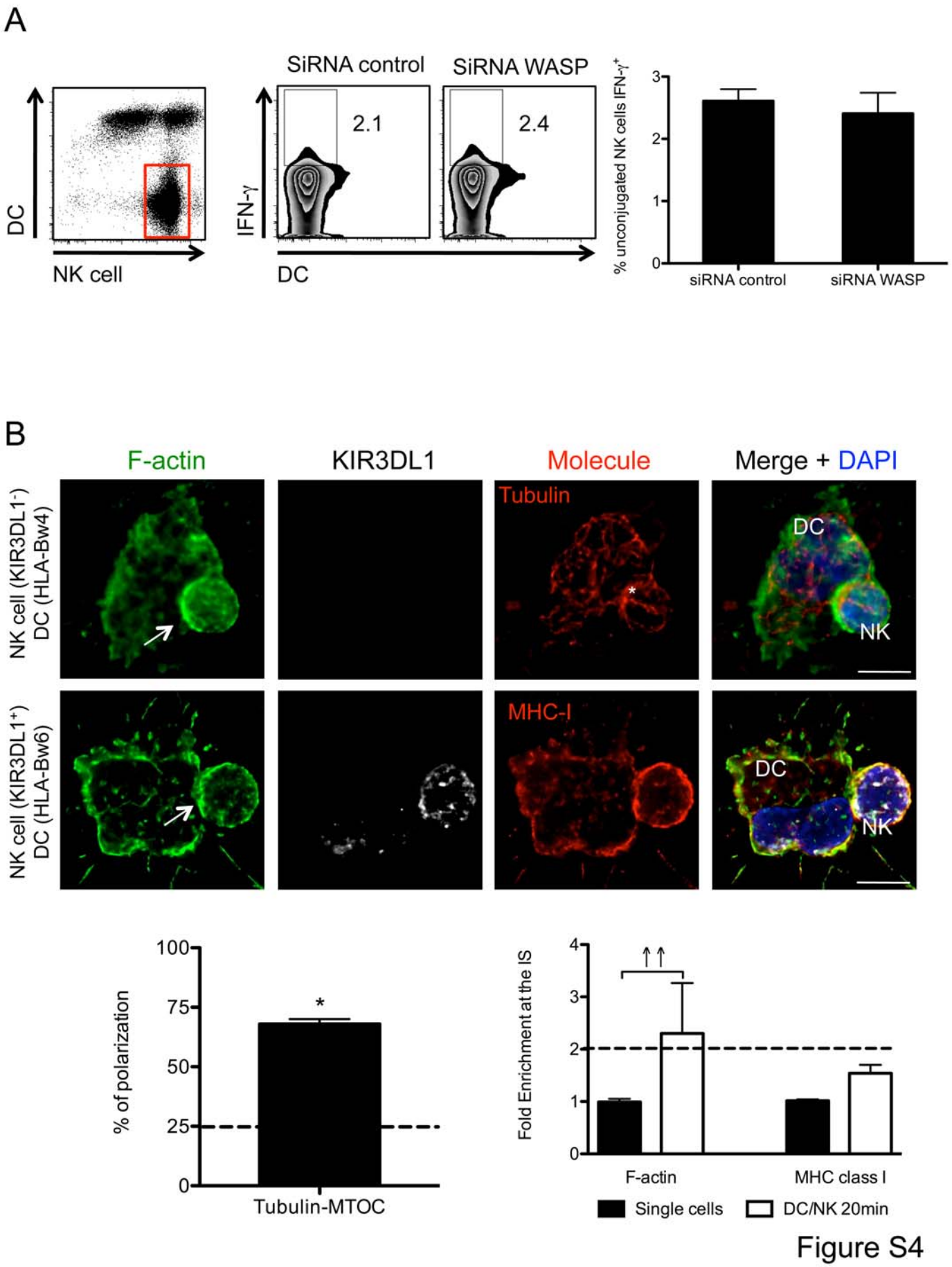
A
SiRNA control
SiRNA WASP
2.1
2.4
DC
NK cell
IFN-γ
DC
% unconjugated NK cells IFN-γ+
siRNA control
siRNA WASP
B
F-actin
KIR3DL1
Molecule
Merge + DAPI
NK cell (KIR3DL1-)
DC (HLA-Bw4)
Tubulin
DC
NK
NK cell (KIR3DL1+)
DC (HLA-Bw6)
MHC-I
DC
NK
% of polarization
Tubulin-MTOC
Fold Enrichment at the IS
F-actin
MHC class I
Single cells
DC/NK 20min

Figure S4

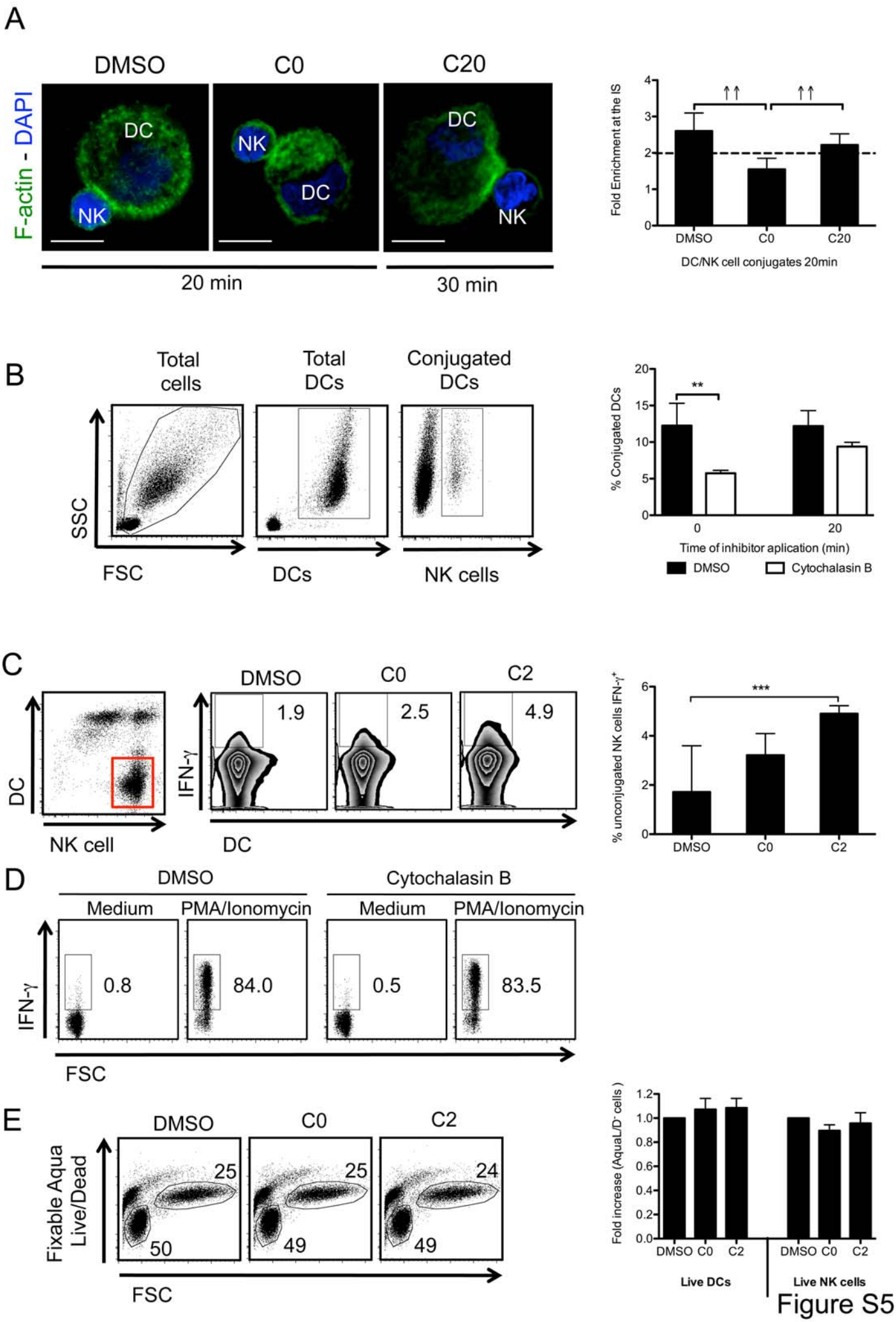
A
DMSO
C0
C20
F-actin - DAPI
DC
NK
20 min
30 min
Fold Enrichment at the IS
DMSO
C0
C20
DC/NK cell conjugates 20min
B
Total cells
Total DCs
Conjugated DCs
SSC
FSC
DCs
NK cells
% Conjugated DCs
Time of inhibitor aplication (min)
DMSO
Cytochalasin B
C
DMSO
C0
C2
1.9
2.5
4.9
DC
NK cell
IFN-γ
% unconjugated NK cells IFN-γ+
D
DMSO
Cytochalasin B
Medium
PMA/Ionomycin
0.8
84.0
0.5
83.5
IFN-γ
FSC
E
Fixable Aqua Live/Dead
25
50
25
49
24
49
Fold increase (AquaL/D- cells)
Live DCs
Live NK cells

Figure S5

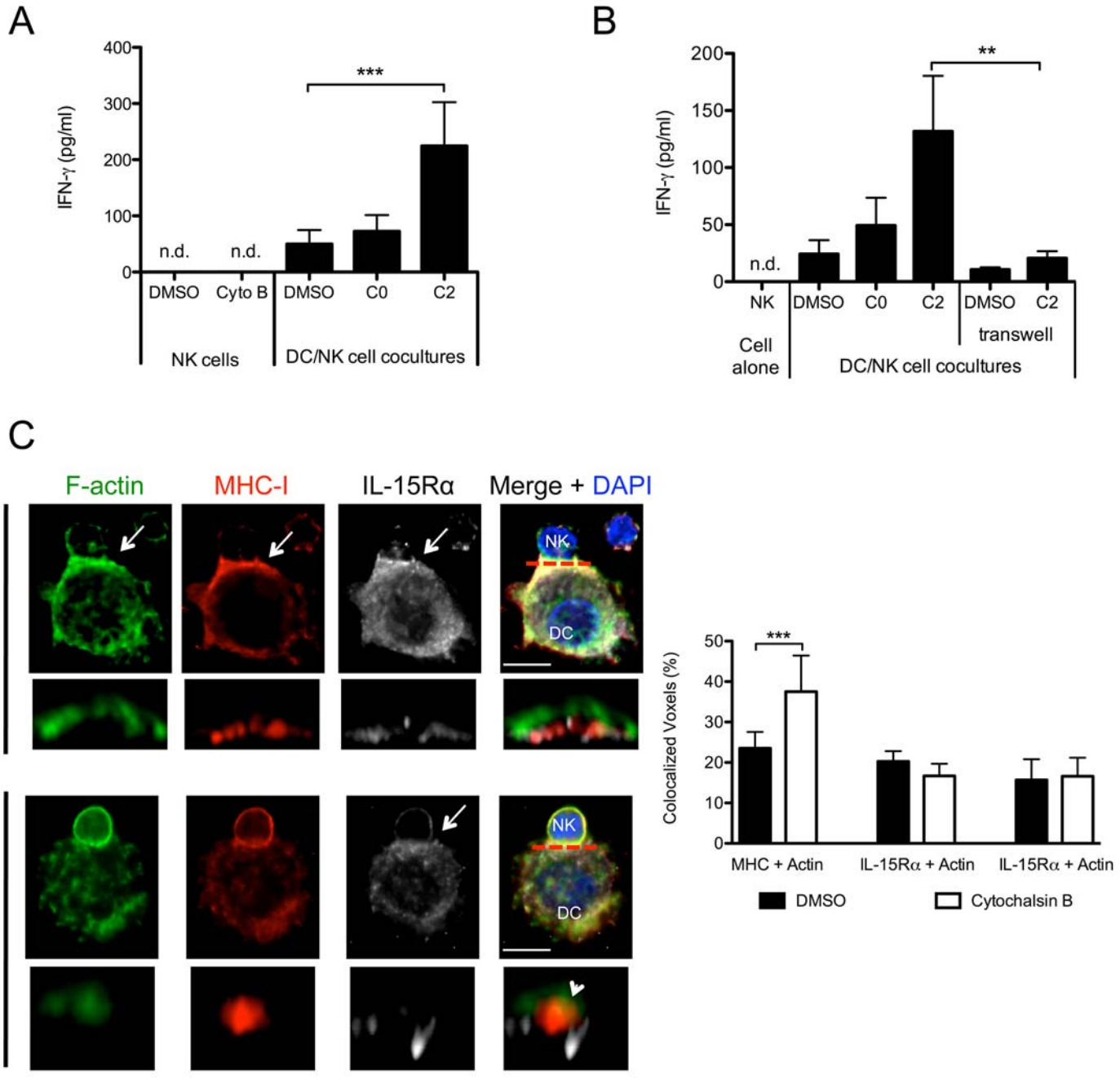
A
IFN-γ (pg/ml)
400
300
200
100
0
***
n.d.
n.d.
DMSO
Cyto B
DMSO
C0
C2
NK cells
DC/NK cell cocultures
B
IFN-γ (pg/ml)
200
150
100
50
0
**
n.d.
NK
DMSO
C0
C2
DMSO
C2
transwell
Cell alone
DC/NK cell cocultures
C
F-actin
MHC-I
IL-15Rα
Merge + DAPI
DMSO
Cytochalasin B
NK
DC
Colocalized Voxels (%)
50
40
30
20
10
0
***
MHC + Actin
IL-15Rα + Actin
IL-15Rα + Actin
DMSO
Cytochalsin B
DC/NK cell conjugates 20min

Figure S6

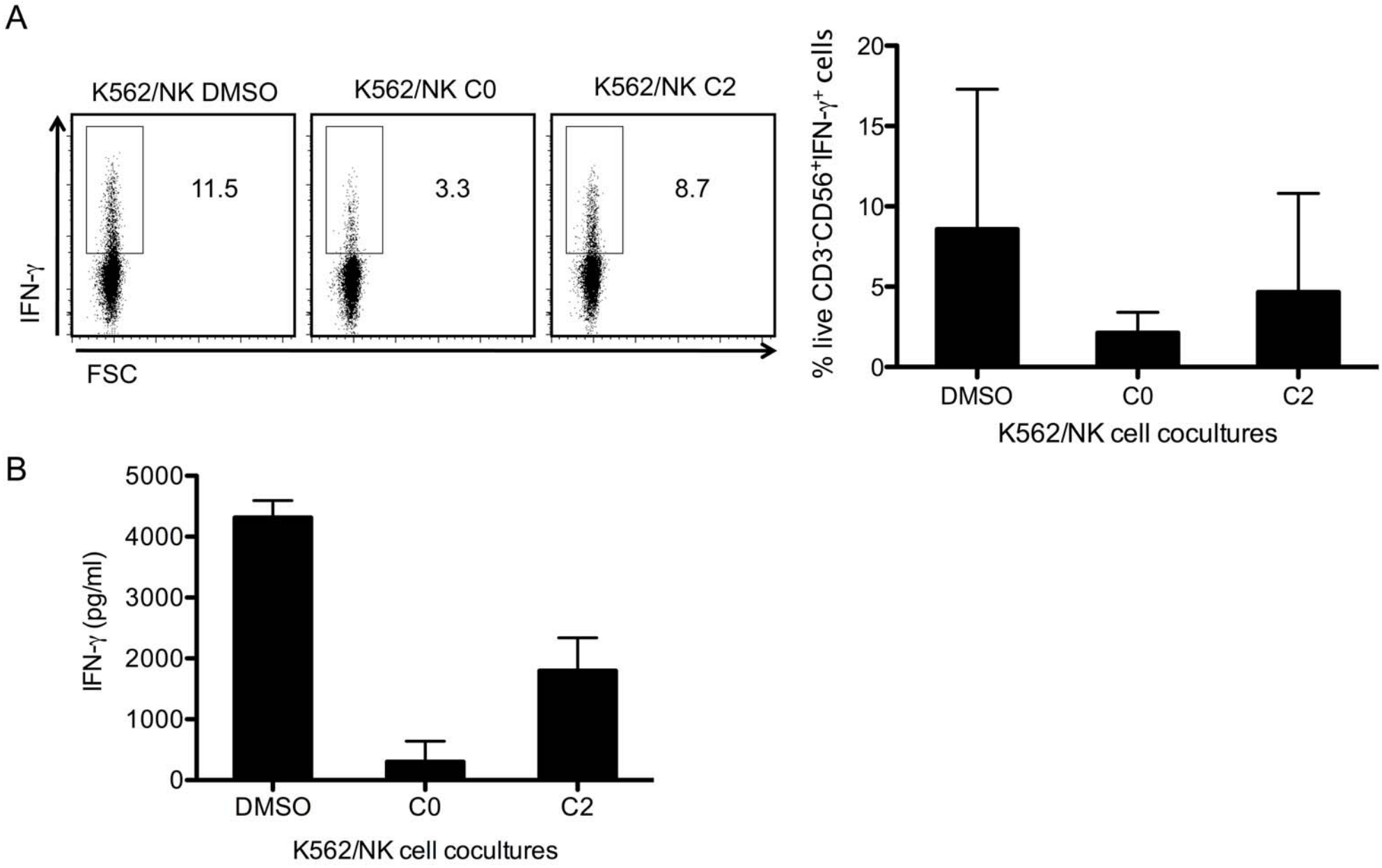
A
K562/NK DMSO
K562/NK C0
K562/NK C2
11.5
3.3
8.7
IFN-γ
FSC
% live CD3-CD56+IFN-γ+ cells
20
15
10
5
0
DMSO
C0
C2
K562/NK cell cocultures
B
IFN-γ (pg/ml)
5000
4000
3000
2000
1000
0
DMSO
C0
C2
K562/NK cell cocultures

Figure S7